Fasciola hepatica from naturally infected sheep and cattle in Great Britain are diploid

N.J. BEESLEY[1*], K. CWIKLINSKI[1], D. J. L. WILLIAMS[1] and J. HODGKINSON[1]

[1] *Veterinary Parasitology, Institute of Infection and Global Health, University of Liverpool, Liverpool, L3 5RF, UK*

Ploidy of wild British *Fasciola hepatica* populations

*Corresponding author: Veterinary Parasitology, Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool, L3 5RF, UK

Tel: +44 (0)151 7950235. Email: nbeesley@liv.ac.uk.

SUMMARY

Diploid (2n=2x=20) and triploid (2n=3x=30) *Fasciola hepatica* have been reported in the UK, and in Asia diploid, triploid and mixoploid (2x/3x) *Fasciola* spp. exist but there is little information to indicate how common triploidy is, particularly in UK fluke. Here the ploidy of 565 adult *F. hepatica* from 66 naturally infected British sheep and 150 adult *F. hepatica* from 35 naturally infected British cattle was determined. All 715 of these parasites were diploid, based on observation of ten bivalent chromosomes and sperm (n=335) or, since triploids are aspermic, sperm alone (n=380). This constitutes the first extensive analysis of the ploidy of *F. hepatica* field isolates from Great Britain and shows that most *F. hepatica* isolated from cattle and sheep are diploid and have the capacity to sexually reproduce. These data suggest that triploidy, and by extension parthenogenesis, is rare or non-existent in wild British *F. hepatica* populations. Given that *F. hepatica* is the only species of *Fasciola* present in Britain our results indicate that the parasite is predominantly diploid in areas where *F. hepatica* exists in isolation and suggests that triploidy may only originate in natural populations where co-infection of *F. hepatica* and its sister species *Fasciola gigantica* commonly occurs.

Key words: *Fasciola hepatica*, ploidy, population genetics, diploid, triploid

KEY FINDINGS

- *F. hepatica* were collected from naturally infected sheep and cattle from British abattoirs
- The criteria for determining ploidy was chromosomal enumeration or observation of sperm
- Ploidy was determined in 715 wild British *F. hepatica*. All were diploid and contained sperm
- The proportion of triploids is 0% (95% CI 0 – 0.49%)
- The spermic and diploid nature of *F. hepatica* facilitates downstream genetic studies

41 INTRODUCTION

42

43 *Fasciola hepatica* is a digenean parasite that causes disease of economic importance in sheep and
44 cattle, and as a zoonosis it is classed by the World Health Organisation as a neglected tropical
45 disease (Hopkins, 1992; Rim *et al.*, 1994; Hillyer and Apt, 1997; WHO, 2015).

46 Reports of the ploidy of *F. hepatica* vary, yet determining ploidy is important to enhance
47 our understanding of parasite biology and is essential to increase our knowledge of parasite
48 genetics, reproduction and the level of gene flow from one population to another. Such studies are
49 necessary to better understand the genetic evolution and spread of drug resistance within parasite
50 populations. Knowledge of ploidy is also vital for facilitating our understanding of genetic diversity
51 of *F. hepatica* populations, genome assembly (Cwiklinski *et al.*, in press) and for design of gene
52 knockdown experiments in downstream functional analyses such as RNA interference (McGonigle
53 *et al.*, 2008).

54 In the UK, *F. hepatica* was first reported to be diploid with 10 bivalent chromosomes (2n =
55 2x = 20) by Sanderson (1953). Since then there have been several reports describing fluke with 10
56 bivalent pairs of chromosomes including the triclabendazole resistant laboratory isolate of *F.*
57 *hepatica* Sligo (n = 5) originally isolated from sheep in the Republic of Ireland (Fletcher *et al.*,
58 2004), wild-type *F. hepatica* (n = 15) from cattle livers in Northern Ireland (Fletcher *et al.*, 2004)
59 and *F. hepatica* (n = 10) obtained from a cattle liver in Slovakia (Reblánová *et al.*, 2011). In
60 contrast, the triclabendazole susceptible laboratory isolate Cullompton (n = 10), originally isolated
61 from sheep in the UK, was shown to be triploid with 30 univalent chromosomes (2n = 3x = 30;
62 Fletcher *et al.*, 2004).

63 To analyse ploidy in *F. hepatica*, the rapidly dividing sperm cells can be used to visualise
64 chromosomes. Spermatogenesis in *F. hepatica* follows a series of three mitotic divisions (from one
65 primary spermatogonia to two secondary spermatogonia to four tertiary spermatogonia to an 8-cell
66 rosette of primary spermatocytes) followed by two meiotic divisions to produce 16 secondary

spermatocytes and then 32 haploid spermatozoa (John, 1953; Gresson, 1965; Stitt and Fairweather, 1990). The triploid Cullompton parasites were aspermic due to a failure of the first meiotic division (Fletcher *et al.*, 2004), and were therefore assumed to undergo parthenogenesis to reproduce (Hanna *et al.*, 2008).

Most of the studies on Asian *Fasciola* spp. do not differentiate between *F. hepatica* and its sister species *Fasciola gigantica* because intermediate forms of the parasite exist (Itagaki *et al.*, 1998; Terasaki *et al.*, 2000; Itagaki *et al.*, 2009; Peng *et al.*, 2009). Diploid and triploid *Fasciola* spp. have been isolated from both cattle and deer livers in Japan (1 diploid and 18 triploid; Terasaki *et al.*, 1998; 2000; 3 triploid; Itagaki *et al.*, 1998; 1 diploid and 11 triploid; Itagaki *et al.*, 2005a); from cattle livers in Vietnam (19 diploid and 22 triploid; Itagaki *et al.*, 2009); and from cattle livers in Korea (65 diploid and 19 triploid; Terasaki *et al.*, 2000; 1 diploid and 1 triploid; Itagaki *et al.*, 2005b). In Korea an additional level of complexity has also been reported as in addition to diploid (n = 143) and triploid (n = 23) *Fasciola* spp., mixoploid (n = 46), also called mosaic or chimera, *Fasciola* spp. were found. The mixoploid parasites have a mixture of cells, some with a diploid number of chromosomes and some with a triploid number (2x/3x; Rhee *et al.*, 1987). Usually the Asian triploids showed abnormal spermatogenesis (Itagaki *et al.*, 1998; 2009). However diploid *Fasciola* spp. with no sperm and triploid *Fasciola* spp. with small numbers of sperm have also been found (Terasaki *et al.*, 1998; 2000).

To date, studies on the ploidy of *F. hepatica* in the UK have been limited to small numbers (5-15 parasites; Fletcher *et al.*, 2004). Due to the differing reports of ploidy in this parasite, here we determined the ploidy of a larger number of *F. hepatica* isolated from naturally infected sheep and cattle in Great Britain. The results of this study may also be relevant to other areas where *F. hepatica* exists in isolation from *F. gigantica*.

MATERIALS AND METHODS

Fasciola hepatica *collection*

Fasciola hepatica was recovered from the livers of 66 naturally infected lambs between November 2012 and February 2013, from three different abattoirs located in Wales, North West England and Central England. Based on the catchment areas of these abattoirs, these samples represent field populations of *F. hepatica* from England, Wales and Scotland. *Fasciola hepatica* was recovered from 35 cattle livers between October 2013 and January 2014, from an abattoir located in Wales. Based on ear tag information, these samples represent field populations of *F. hepatica* from England and Wales, and the cattle were a mixture of beef and dairy breeds with a mean age of 7.4 years (range 1.6 to 15.1).

Sheep livers were transported to the University of Liverpool, where adult parasites were isolated from the bile ducts. Parasites from cattle livers were isolated *in situ* at the abattoir. To purge intestinal contents and eggs, the parasites were incubated, for a minimum of 2h at 37^{o}C in 1-2ml of Dulbecco's Modified Eagle's Media (Sigma-Aldrich) with 120µg/ml of gentamicin (Sigma-Aldrich) and 120µg/ml of amphotericin B (Sigma-Aldrich). After incubation, each individual parasite was washed in Dulbecco's Phosphate Buffered Saline (PBS). A total of 565 *F. hepatica* were recovered from sheep with one to twelve parasites obtained from each liver, and 150 *F. hepatica* were recovered from cattle with one to seven parasites from each liver.

Determining ploidy of Fasciola hepatica

An additional 129 *F. hepatica* collected from sheep were used to optimise methods of chromosome visualisation adapted from Fletcher *et al.* (2004) and Reblánová *et al.* (2011). Whilst it was preferable to use fresh material and perform an aceto-orcein squash immediately after a 2h purge of adult *F. hepatica*, the practicalities of using large numbers of parasites necessitated purging for 2h followed by incubation in 0.025% (w/v) colchicine (Sigma-Aldrich) in PBS, pH 7.4 for 1h at RT, followed by incubation in 75mM potassium chloride (VWR) for 1h at RT, and fixing (3:1, ethanol: acetic acid). Aceto-orcein squash could then be performed at a later date. Briefly, for aceto-orcein

squash a section of the distal two thirds of the parasite (approximately 1mm^3) was macerated in a drop of 3% (w/v) orcein (Sigma-Aldrich) in 45% (w/v) acetic acid on a glass slide, and then squashed under a cover slip using filter paper to soak up the excess stain. All the samples of *F. hepatica* from sheep were examined in this manner but all parasites from cattle were examined immediately after purging i.e. using fresh material.

Preparations were examined using a Zeiss Axio Imager M2 microscope and Zen 2011 software. The stages of spermatogenesis were identified: rosettes (8-cell stage); 16-cell stage; 32-cell stage and spermatids and sperm (Fig. 1). The number of chromosomes was counted in well-spread cells (Fig. 2); they were most clearly identified in the first meiotic division from the rosette to the 16-cell stage. Triploid *F. hepatica* is aspermic or have very few abnormally developed sperm, and do not undergo the same stages of spermatogenesis as diploid parasites (Terasaki *et al.*, 2000; Fletcher *et al.*, 2004; Hanna *et al.*, 2008; Itagaki *et al.*, 2009). In previous studies the ploidy of *F. hepatica* was determined at the same time as determining the presence or absence of sperm (Fletcher *et al.*, 2004; Hanna *et al.*, 2008; Itagaki *et al.*, 2009). Therefore, here the presence of sperm was used as a proxy to determine diploidy and a parasite was deemed to be diploid if either 10 bivalent chromosomes or sperm were observed.

Statistical programs

The StatCalc function of Epi Info 7 (http://wwwn.cdc.gov/epiinfo/7/) was used to calculate sample size. Calculations were initially based on an expected proportion of 50% triploid individuals (to give the highest possible sample size required). A sample size of 384 individuals gave 95% confidence level with 5% confidence limits (precision).

Ethical approval

Ethical approval was received from the University of Liverpool's Veterinary Research Ethics Committee (VREC106 and VREC145).

RESULTS

Ploidy was determined for 565 and 150 of the *F. hepatica* parasites isolated from naturally infected sheep and cattle respectively. All 715 parasites, where ploidy was determined, were diploid. The proportion of triploids identified was 0% (95% CI 0 – 0.49%; Hanley and Lippman-Hand, 1983). No difference between the ploidy of parasites isolated from sheep and cattle was found.

Ten bivalent chromosomes (Fig. 2) and sperm were observed in 335 (46.9%) of all *F. hepatica* samples; sperm alone were observed in 380 (53.1%) of the samples. Different stages of spermatogenesis: rosettes (8-cell stage); 16-cell stage; 32-cell stage and spermatids and/or sperm were observed in 93.3%, 34.7%, 72.9% and 100% of samples, respectively (Fig. 1; Table 1). All stages of spermatogenesis were observed in 144 *F. hepatica* from sheep and 53 *F. hepatica* from cattle. Sperm cells were commonly associated with the rosette and 32-cell stages; fewer 16-cell stages were observed. If the 16-cell stages were excluded, all other stages of spermatogenesis (rosette, 32-cell and sperm) were seen in 360 *F. hepatica* from sheep and 146 from cattle (Table 1).

DISCUSSION

The *F. hepatica* samples analysed in this study were isolated from naturally infected sheep and cattle from England, Wales and Scotland. All 715 parasites, where ploidy was determined, were diploid as shown by detection of 10 bivalent chromosomes, or sperm. These results are consistent with other studies of ploidy in wild populations of *F. hepatica*. In Northern Ireland a smaller scale study identified diploid parasites (n = 15) isolated from naturally infected cattle (Fletcher *et al.*, 2004) and similarly a study in Slovakia which analysed 10 parasites from a cattle liver found exclusively diploid organisms (Reblánová *et al.*, 2011). Overall the results from this study suggest

that triploidy in *F. hepatica* populations, at least in Great Britain, is a rare occurrence. The presence of sperm in all of the mature parasites studied here is in contrast to *Fasciola* spp. in Asian populations which have frequently been described as aspermic or, triploid and aspermic, and are assumed to reproduce by parthenogenesis (Itagaki *et al.*, 1998; 2009; Terasaki *et al.*, 1998; 2000). The results here suggest that sexual reproduction, rather than parthenogenesis, is the most frequent means of reproduction in *F. hepatica* populations from Great Britain.

Different isoenzyme electrophoretic patterns provide evidence that triploidy in Japanese *Fasciola* spp. has arisen independently on more than one occasion (Agatsuma *et al.*, 1994). Sequence comparison of the mitochondrial NADH dehydrogenase subunit I (NDI) and cytochrome *c* oxidase subunit I (COI) from triploid *Fasciola* spp. from Japan showed identity to *F. gigantica* from Zambia but were different to *F. hepatica* from Uruguay (Itagaki *et al.*, 1998). However, when comparing the ribosomal DNA internal transcribed spacer (ITS) 2 sequence, six of seven Japanese triploids were almost identical to *F. hepatica* from Uruguay, whilst one parasite showed greater identity to *F. gigantica* from Indonesia (Itagaki and Tsutsumi, 1998). Interestingly chimeric sequences (with nucleotide regions common to *F. hepatica* and *F. gigantica*) have been observed in the ITS-2 of Korean *Fasciola* spp. (Agatsuma *et al.*, 2000) as well as the ITS-1 of triploid *Fasciola* spp. from Vietnam and aspermic *Fasciola* spp. from China and Bangladesh (Itagaki *et al.*, 2009; Peng *et al.*, 2009; Mohanta *et al.*, 2014). The majority of sequencing evidence therefore supports the hypothesis that triploids are the result of hybridisation between *F. hepatica* and *F. gigantica* in areas where their distribution overlap. Since *F. hepatica* and *F. gigantica* have been found in the same definitive host (Amer *et al.*, 2011), this presents the opportunity for hybridisation between the two species and confirmation of the ability of *F. hepatica* and *F. gigantica* to cross-fertilise using experimental systems supports this hypothesis (Itagaki *et al.*, 2011).

There is one report of triploidy in *F. hepatica* in the UK, in the Cullompton laboratory isolate. This isolate was derived in 1998 from multiple eggs obtained from the bile ducts of several sheep, which were used to infect snails, and then passaged twice through sheep and once through

197 rats. The analysis of ploidy on this isolate was not performed until between 2001 and 2003 (Fletcher
198 *et al.*, 2004). Since UK populations of *Fasciola* spp. are restricted to *F. hepatica* it is unlikely that
199 triploids formed by hybridisation, and our results suggest that triploidy in the Cullompton isolate is
200 an artefact of isolation and laboratory passage of this isolate, rather than being a true reflection of
201 what is occurring in the field. It is reported the Cullompton isolate was passaged several times
202 following isolation; potentially creating a bottleneck. Passage was also through rats, a non-natural
203 host of *F. hepatica* with far smaller livers able to support far fewer individual fluke, compared to
204 sheep and cattle, which may have increased stress on the parasites. Whilst we do not know
205 definitively the effect long term laboratory passage has had on the parasite, these stresses may have
206 led to the induction of triploidy in the Cullompton isolate. It is possible to induce polyploidy in
207 helminths: heat shock induced polyploidy in *Caenorhabditis elegans* producing tetraploids from
208 diploids, probably via a triploid (Madl and Herman, 1979). Other stressors have also been shown to
209 alter ploidy in vertebrates. For example in bovine embryos the chemical cytochalasin B induced
210 changes in ploidy (Bai *et al.*, 2011), and in leopard frogs, *Rana pipiens*, hydrostatic pressure has a
211 similar affect (Dasgupta, 1962). In plant species, wounding and water or nutrient stress have also
212 been shown to alter ploidy (Ramsey and Schemske, 1998). If one of these stressors produced a
213 diploid female gamete, a triploid would be produced following fertilisation with a haploid sperm. In
214 order to maintain triploidy over successive generations, the initial creation of an accidental triploid
215 would have to be derived from a parthenogenic diploid (Terasaki *et al.*, 2000). Fletcher *et al.* (2004)
216 also suggested that triploidy could be produced by the fertilisation of a haploid egg with two
217 haploid sperm. However, in another study of *F. hepatica*, whilst more than one sperm became
218 enclosed within the capsule of the egg, more than one sperm was never observed in the ooplasm
219 (Sanderson, 1959). A further possibility is the introduction of triploids, from an endemic area into
220 the UK. Although there is evidence that triploid *Fasciola* spp. can stabilise within a population once
221 introduced (Itagaki *et al.*, 2005a), the UK does not routinely import from such endemic areas.

Despite being triploid and aspermic, the infectivity of the Cullompton isolate has not been affected, since recent experiments have demonstrated that the isolate will successfully infect sheep and cattle (Hanna *et al.*, 2008). However laboratory isolates are often not representative of what is happening in the field and knowledge of the provenance of isolates, their maintenance within the laboratory in terms of passage and continued assessment of their infectivity, pathogenicity and resistance status is an essential part of studies on *F. hepatica* but is frequently overlooked (Hodgkinson *et al.*, 2013).

Since the study reported here has shown that all the *F. hepatica*, from naturally infected sheep and cattle in Great Britain, were diploid, triploidy, if it exists, is rare. These results suggest that triploidy is most likely to develop from hybridisation between *F. hepatica* and *F. gigantica*. If this is the case, it means that the results of this study may be extrapolated to other countries where, like the UK, the only species present is *F. hepatica*. This, taken with the observation that sexual reproduction was more frequent than parthenogenesis (since all the parasites studied here contained sperm) will enable further genetic and molecular studies of *F. hepatica* populations. Knowledge that *F. hepatica* populations in the UK are diploid facilitates the successful silencing of target genes using RNA interference (McGonigle *et al*., 2008) and is essential to support assumptions about inheritance patterns and for the calculation of observed and expected allele frequencies that are fundamental to population genetic studies (Dufresne *et al*., 2014). Most recently the diploid nature of *F. hepatica* was an important consideration in the assembly of our 1.3Mb genome (Cwiklinski *et al.*, in press).

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Table 1. Ploidy of wild British *Fasciola hepatica* from sheep and cattle, a breakdown of the spermatic stages observed and details of parasite burden

Species	No. of Animals	Median adult *Fasciola hepatica* per liver (range)	No. of *F. hepatica* analysed (range per liver)	No. of parasites in which each stage of spermatogenesis was observed (%)				No. of parasites with 10 bivalent chromosomes (%)	No. of diploid *F. hepatica* (%)
				8 cells	16 cells	32 cells	sperm		
Sheep	66	10*	565	517	194	375	565	246	565
		(1 – >100)	(1 – 12)	(91.5)	(34.3)	(66.4)	(100)	(43.5)	(100)
Cattle	35	15	150	150	54	146	150	89	150
		(1 – 133)	(1 – 7)	(100)	(36)	(97.3)	(100)	(59.3)	(100)
Total	101	11.5*	715	667	248	521	715	335	715
				(93.3)	(34.7)	(72.9)	(100)	(46.9)	(100)

*Total enumeration of parasites was not determined for 19 of the sheep livers, therefore these figures are based on 47 of the 66 sheep

Fig. 1: The different stages of spermatogenesis in *Fasciola hepatica*: (A) the 8-cell or rosette stage; (B) the 16-cell stage; (C) the 32-cell stage (black arrows); (D) the nuclei from the 32-cell stage elongate to spermatids (black arrow) and then sperm (white arrows)

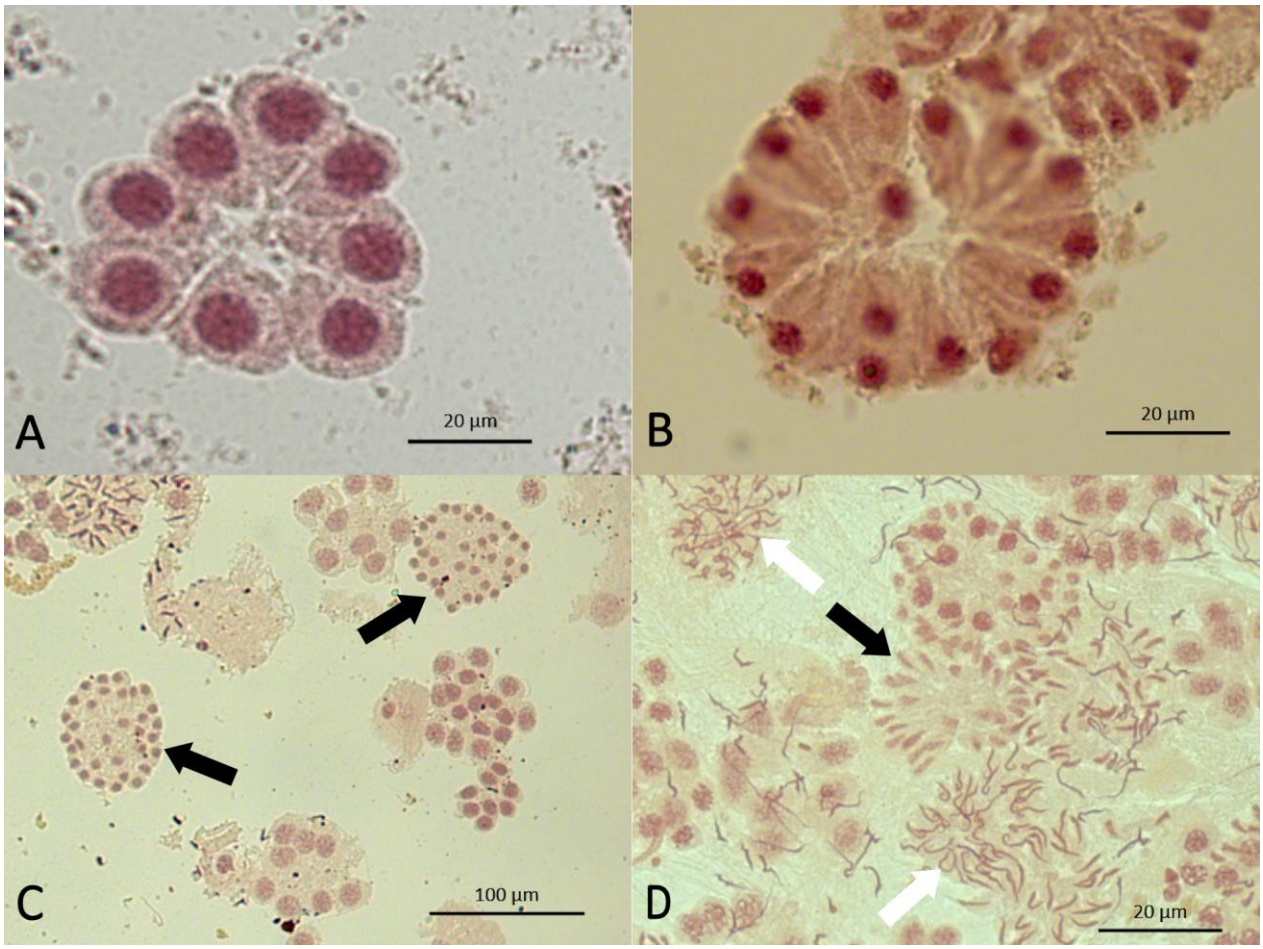

Fig. 2: Examples of chromosomes from *Fasciola hepatica*: in well-spread cells (arrows) chromosomes can be counted: (A) *F. hepatica* from a sheep liver; (B) *F. hepatica* from a cow liver